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Rapid report

Quantitative analysis of mitochondrial DNA deletion in paraffin embedded muscle tissues from patients with KSS and CPEO

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Abstract

The percentage of common deletion of mitochondrial DNA (mtDNA) was determined quantitatively by a PCR-based, non-radioactive method in DNA extracted from formalin-fixed, paraffin-embedded skeletal muscle tissues from two patients with Kearns Sayre syndrome (KSS) and one with chronic progressive external ophthalmoplegia (CPEO). The method involved PCR cycle titration of wild-type and deleted mtDNA in parallel, staining of gel bands with the sensitive fluorescence dye SYBR Green I, and quantitation of intensity on a computer screen by the NIH image program. We determined 75% and 71% common deletion of mtDNA in the KSS patients and 35% in the CPEO patient.

Keywords: Mitochondrial DNA; Common deletion; Quantitative PCR; Mitochondrial myopathy

The aim of the present investigation was the quantitative determination of the common deletion of mitochondrial DNA (mtDNA) in formalin-fixed, paraffin-embedded muscle tissues from patients with Kearns Sayre syndrome (KSS) and chronic progressive external ophthalmoplegia (CPEO). Determination of mutations of mtDNA in paraffin-embedded tissues by PCR-based methods is possible, if the amplified fragments are small [1–3], because the DNA in paraffin blocks is partially degraded into smaller fragments. In previous studies the amount of PCR-amplified mtDNA fragments was determined either by using radioactivity [4,5], or by densitometry of gel bands, stained with ethidium bromide, using a gel scanner [2]. Here we use three primers to amplify in parallel wild-type and deleted mtDNA [4]. Quantitation of amplified mtDNA fragments was made without using radioactivity by staining gel bands with the sensitive fluorescence dye SYBR green I, recording the image with a video camera, and analyzing the intensity on the computer screen using the NIH image program, as previously described [6].

The diagnosis of Korean patients was made by clinical presentations, neurological examinations combined with enzyme histochemistry and characteristic electron microscopic findings (data not shown). Deltoid muscle biopsy specimens from a 22-year-old female (patient 1) and a 20-year-old male with KSS (patient 2), and calf muscle specimens of a 13-year-old male with CPEO (patient 3), were formalin-fixed and embedded in paraffin after histochemical and ultrastructural analysis.

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DNA was extracted from paraffin blocks with xylene by a modified method of Kösel and Graeber [1]. Small amounts of tissue were cut from paraffin blocks using a sterile scalpel blade and incubated with 400 μl xylene and 500 μl mineral oil for 2 h at room temperature (RT) under gentle agitation. After centrifugation (5 min, 12 000 × g), the paraffin-containing supernatant was discarded, and the extraction was repeated with 400 μl xylene. In order to remove remaining xylene, the pellet was again extracted with 400 μl ethanol. After solubilization in 140 μl PCR-TE buffer (10 mM EDTA, 200 mM Tris-HCl, pH 8.0), 20 μl 10% SDS and 80 μl 1% proteinase K were added and incubated overnight at 55°C. DNA was further purified by phenol/chloroform extraction, and the resulting pellets were resuspended in 50 μl PCR-TE buffer and used for PCR.

Control DNA was extracted according to the method of Wallace et al. [7] from a postmortem skeletal muscle sample of a 79-year-old healthy individual, obtained within 24 h after death. A plasmid standard for the common deletion of mtDNA (pCII) was a gift of Prof. Eric A. Schon, Columbia University, New York, containing a 505 bp PCR-amplified mtDNA fragment, lacking the 4977 bp common deletion [8].

In order to identify wild-type mtDNA and the common deletion of mtDNA in parallel [4], PCR was carried out using the forward primer P1 (nt 8421–8440, 5′-CACTATTCCTCATCACCCAA-3′) and reverse primer P2 (nt 8573–8557, 5′-ATGTGGTCTTTGGAGTAGAA-3′) for a 152 bp wild-type mtDNA fragment, and P1 and the reverse primer P3 (nt 13520–13501, 5′-CCTAGGATTGTGGGGGC-3′) for a 123 bp fragment characteristic for the 'common deletion' of mtDNA. PCR amplifications were carried out in a volume of 50 μl using 20 μM of each dNTP, 0.5 μl or 1 μl Vent polymerase (2000 units/ml) (New England, Biolabs), 2 mM $MgSO_4$ and 30 pM of each of the three primers overlaid with 35 μl wax. After a single predenaturation step (5 min at 95°C) PCR was done by 1 min denaturation at 94°C, 1 min annealing at 56°C, and 1 min elongation at 72°C for the indicated number of cycles in a Perkin-Elmer PCR-Thermal Cycler.

Fig. 1 shows PCR amplified DNA fragments of 152 bp (wild-type mtDNA) and 123 bp (deleted mtDNA) of patient 1 (lanes 3–6), patient 2 (lanes 7–10), and patient 3 (lanes 11–14), obtained after 25, 27, 29 and 31 cycles, respectively. In the case of the two KSS patients (lanes 3–10) the fragments of deleted mtDNA were more intensive than those of wild-type mtDNA. In contrast, with the CPEO patient (lanes 11–14), the wild-type mtDNA fragment was more intensive than that of deleted mtDNA.

In Fig. 2 is shown the intensity of gel bands of wild-type and deleted mtDNA of four different PCR cycles with the DNA of patient 1, carried out with two concentrations of DNA polymerase. Quantification of gel bands, recorded by a video camera and analyzed by the NIH image program on a computer, resulted in saturation curves for the wild-type and deleted fragment. Calculation of the percentage of deleted mtDNA, however, resulted in decreasing values with increasing PCR cycles. This result did not change when the amount of Vent polymerase was

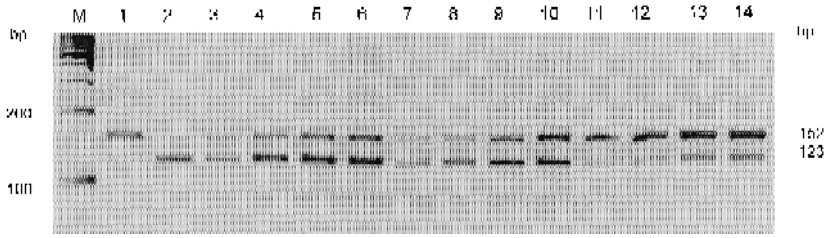

Fig. 1. PCR amplification of wild-type and deleted mtDNA in DNA samples extracted from paraffin-embedded skeletal muscles from patients with KSS and CPEO. The 4% Nusieve GTG agarose (FMC, Rockland, ME, USA) gel was stained with SYBR Green I (Molecular Probes, Eugene, Oregon, USA) and the image recorded with a CCD video camera VarioCam (Labortechnik Fröbel, Wasserburg, Germany). M: Molecular weight marker (BioLadder™ 100, AGS GmbH, Germany). Lane 1: Control DNA of a 79-year-old healthy individual; lane 2: standard DNA for the common deletion of mtDNA (pCII); lanes 3–6: patient 1; lanes 7–10: patient 2; lanes 11–14: patient 3. Lanes 3, 7, 11: 25 cycles; lanes 4, 8, 12: 27 cycles; lanes 5, 9, 13: 29 cycles; lanes 6, 10, 14: 31 cycles.

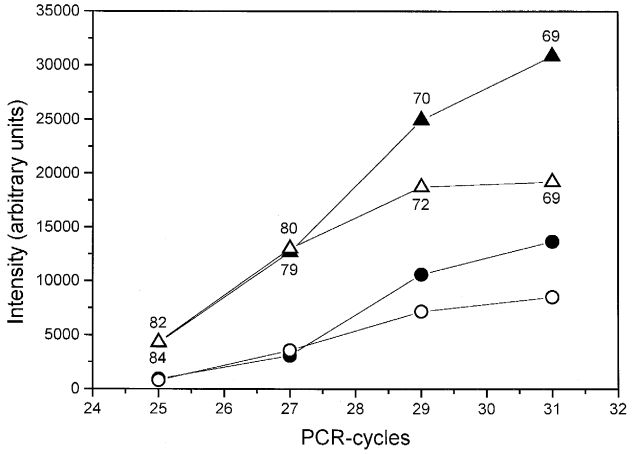

Fig. 2. Intensities of gel bands of PCR-amplified wild-type (circles) and deleted mtDNA (triangles) of KSS patient 1 at increasing numbers of PCR cycles (lanes 3–6 of Fig. 1). The fluorescence intensities of gel bands, recorded with a CCD-video camera VarioCam, connected with a frame grabber, Scion LG3-01 (Labortechnik Fröbel, Wasserburg, Germany), was determined on a computer screen using the public domain MH image program (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov) with the macros Gel_Plotting_Andreas.txt (written by Andreas Becker and available from the same site in the directory puh/hih-image/user-macros) as described before [4]. The numbers in the figure above the triangles indicate the percentage of deleted mtDNA in samples amplified with 1.0 μl polymerase (closed symbols), the numbers below the triangles with 0.5 μl polymerase (open symbols), calculated by correcting the intensities of deleted mtDNA fragments (123 bp) with the factor 152/123.

increased. With double amounts of polymerase, the amounts of amplified fragments increased at higher PCR cycles, but the percentage of deleted mtDNA was almost the same. The true percentage of deleted mtDNA must be calculated from the linear range of PCR cycle titration, i.e. between 27 and 29 cycles. For patient 1 (Fig. 2) 75% of deleted mtDNA was calculated. For the other patient with KSS 71%, and for the CPEO patient 35% of deleted mtDNA were calculated, using the same procedure (not shown). More exact determinations can be made by separate amplification of wild-type and deleted mtDNA and by combination of serial dilution and cycle titration PCR [6]. The described method, however, is more simple and avoids the use of radioactivity, as applied in most previous studies [2,4,5].

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